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TITLE: *Discovery of Host Factors and Pathways Utilized in Hantaviral Infection*

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CONTRACTING ORGANIZATION:
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14. ABSTRACT

Hantaviruses are negative-sense RNA enveloped viruses that are transmitted to humans in aerosols of rodent excreta. Hantaviral infections are associated with two significant human diseases: hemorrhagic fever with renal syndrome (HFRS) which is caused by "Old World" viruses found in Europe and Asia (Hantaan, Seoul, Puumala and Dobrava viruses) or hantavirus pulmonary syndrome (HPS) caused by the "New World" viruses of the Americas (Sin Nombre and Andes viruses). A goal of this project is to utilize high throughput genetic screens to define common cellular pathways, and broadly effective inhibitors targeting these pathways, that impact numerous hantaviruses. In the longer run, we hypothesize that the host factors identified by the proposed research will lead to new druggable targets for combating hantaviral infection.

15. SUBJECT TERMS
Hantavirus, Andes virus, Sin Nombre virus, Puumala virus, Dobrava virus, sterol regulatory complex, haploid genetic screen, cholesterol inhibitors,

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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Table of Contents

1. INTRODUCTION: Hantaviruses are negative-sense RNA enveloped viruses that are transmitted to humans in aerosols of rodent excreta. Hantaviral infections are associated with two significant human diseases: hemorrhagic fever with renal syndrome (HFRS) which is caused by viruses found in Europe and Asia (Hantaan, Seoul, Puumala and Dobrava viruses) or hantavirus pulmonary syndrome (HPS) caused by the "new world" viruses of the Americas (Sin Nombre and Andes viruses). A goal of this project is to utilize a powerful haploid cell genetic screening technology (3,4) to define common cellular pathways, and broadly effective inhibitors targeting these pathways, that impact numerous hantaviruses. In the longer run, we hypothesize that the host factors identified by the proposed research will lead to new druggable targets for combating hantaviral infection.
2. KEYWORDS: Hantavirus, Andes virus (ANDV), Sin Nombre virus (SNV), Puumala virus (PUUV), Dobrava virus (DOBV), Haploid screen, sterol regulatory complex (SRC), haploid genetic screen, cholesterol inhibitors, Major Task (MT).
3. ACCOMPLISHMENTS:

- What were the major goals of the project?
- The major goals of this project are as follows: 1) Analysis of the requirement for cholesterol synthesis by New and Old World hantavirus. 2) Identification and characterization of host factors involved in Old World Hantavirus entry. 3) Identification and characterization of host factors involved in New World Hantavirus entry. Each of these major goals was further divided into tasks and sub-tasks as detailed on the Statement of Work (attached). **In the proposal SOW Major Tasks (MTs) 1, 3 and 4 (portions of Specific Aims 1 & 2) were the focus of year 1.**
- What was accomplished under these goals?

In this this reporting period the majority of the research outlined in the SOW for year 1 was completed for Major Tasks 1 & 4. We have color coded the accomplishments in the attached SOW to denote those that are completed (green), in progress (blue) or have been slightly modified (orange). The work for MT 3 originally slated to be completed in year 1 is in progress but was delayed by a cryogenic storage system failure that resulted in loss of the mutant library (see below). We have regenerated the library (MT3 subtask 1) and completed the selection with rVSV-ANDV (MT3, subtask 2). In contrast we are ahead of schedule and have already begun work on Major Task 5 – work that was initially planned to be completed in year 2. We have completed a majority of the studies of MT5 subtasks 2-5. We have also slightly altered our approach for MT 2 subtasks 1 & 2 and are using a different molecular tag (APEX-Stem in place of HA-Stem) because this new tag provides a more effective system for tracking the viral particles. Again we are ahead of schedule with this work which was initially proposed for year 2. Specific details of the accomplished research is described in the following paragraphs and figures with the Specific Aim and Major Task that the work relates to indicated.

Prior to the start of this award, we identified members of the sterol regulatory complex (SRC) pathway as critical for infection by Andes virus (Petersen et al 2014). As part of Specific Aim 1 (MT1) we sought to determine if a similar requirement exists for New World and Old World hantaviruses.

Figure 1 presents an analysis of the requirement for cholesterol synthesis by prototypic New and Old World hantaviruses. VSV carrying its own glycoprotein is used as a control. Infection mediated by the glycoproteins from both ANDV and PUUV was dramatically impaired in a dose-dependent manner compared to the control.

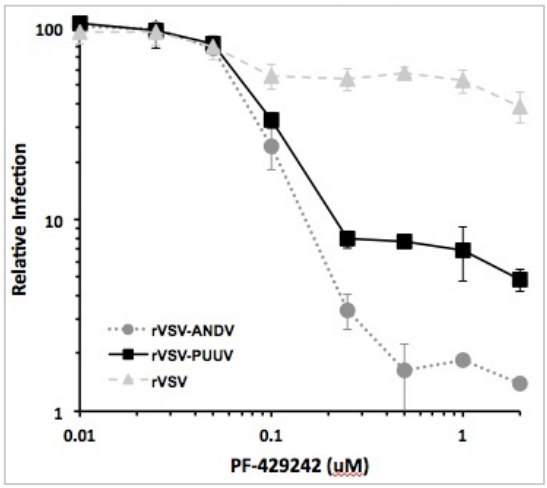
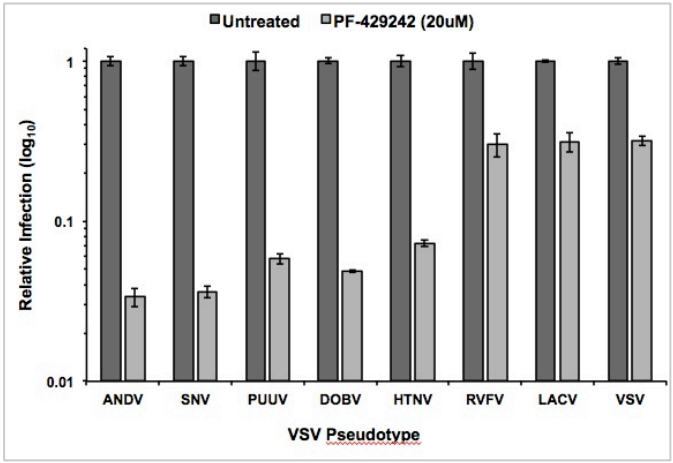


Figure 1. The Sterol Regulatory Complex inhibitor PF-429242 inhibits infection by prototypic New & Old World hantaviruses. *A range of concentrations of the compound PF-429242, which inhibits the Site 1 Protease needed to activate the SRC, was used to treat A549 cells for 12 hours prior to infection with a GFP-expressing VSV vector pseudotyped with the viral glycoproteins from either the New World Andes virus (ANDV) or the Old World Puumala virus (PUUV).*

To extend this finding we analyzed the effect of SRC inhibitors on infection mediated by the glycoproteins for several additional New and Old World hantaviruses as well as more distantly related bunyaviruses, again using VSV glycoprotein as a control (MT1 subtask 3). Figure 2 clearly demonstrates that an additional New World hantaviruses, Sin Nombre (SNV) as well as the Old World hantaviruses (Dobrava; DOBV, Hantaan; HTNV) are impaired similarly to ANDV and PUUV by PF-429242. In contrast the bunyaviruses Rift Valley fever virus (RFTV) and LaCrosse virus (LACV) are minimally impacted by this inhibitor and behave similarly to the control VSV-G. Not shown are additional experiments (MT1 subtask 3) using a cholesterol synthesis inhibitor mevastatin to also deplete cellular cholesterol levels. Again infection mediated by the glycoproteins of both New and Old World hantaviruses is significantly impaired. Thus it appears that a functional cholesterol synthesis pathway is a common requirement for entry by the hantavirus family. Overall, this new work clearly demonstrates that this shared requirement for may present an opportunity to affect all hantaviruses by employing SRC or cholesterol synthesis inhibitors.

Figure 2. PF-429242 inhibits infection mediated by numerous New & Old World hantavirus glycoproteins. *Human A549 cells were treated with 20uM of the compound PF-429242 for 12 hours prior to infection with a GFP-expressing VSV vector pseudotyped with glycoproteins from several hantaviruses. Glycoproteins from distantly related bunyaviruses (RVFV & LACV) Entry by VSV-G glycoprotein was analyzed as a control.*



Additional studies within Specific Aim 1 (Major Task 2) that were initially scheduled to begin in year 2 have already been initiated. These studies will analyze the point at which the SRC is required for hantaviral infection by employing an epitope tagged protein that is co-incorporated along with the hantaviral glycoproteins into the VSV pseudotypes for microscopic analysis of viral entry. Originally, we proposed using a simple protein consisting of a membrane anchor (the VSV-G stem region) with an appended HA epitope tag on the extracellular surface (HA-Gstem). We have modified this plan to include a newly described small peroxidase protein, APEX (2,3), in addition to Gstem and an epitope tag (Figure 3). In addition, 2 other constructs with differing membrane anchoring units were developed with a goal of identifying the one that is most effectively incorporated into virions. We have called these proteins Virion-associated APEX (VaAPEX).

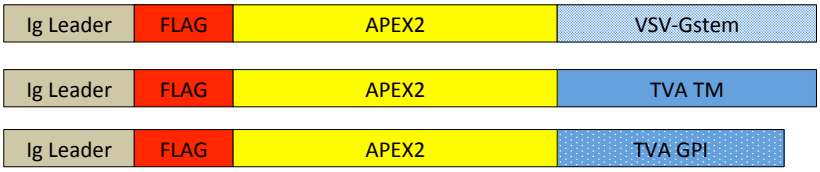


Figure 3. VaAPEX constructs to be employed in analyzing the stage at which cholesterol is needed during hantaviral entry. *These simple proteins consist of a leader sequence (brown), FLAG epitope tag (red), modified ascorbate peroxidase coding region (APEX, yellow), membrane anchor (blue) from either VSV-G, the host protein Tva, or a splice variant of Tva that encodes a GPI-linked lipid anchor*

The APEX technology (2, 3) is based upon the generation of short-lived free radicals that covalently bind electron-rich amino acids in close proximity (~20nm) to the APEX enzyme and can therefore be used to label the area of the cell or cellular compartment in which the incoming virus localizes. Substrates for fluorescent microscopy (as proposed in the original grant) or dense labels for use in electron microscopic analysis are available. The ability to utilize EM or fluorescence microscopy makes this a much more flexible system to determine the point at which the cholesterol pathway is needed in hantaviral infection. To date we have produced three variants of the APEX tagging constructs (Figure 3) and have evaluated them for their ability to incorporate into pseudotyped particles (MT2, subtasks 1 &2). All three are effectively incorporated into VSV, HIV or MLV based viral particles along with hantaviral glycoproteins (MT2, subtask 2, data not shown). We find that transient expression gives much greater incorporation that stable cell lines and will therefore not generate stable Vero lines as was proposed. Moreover APEX is enzymatically active in the released viral particles (data not shown). Thus we have in place and have optimized (MT2, subtask 3) a system to follow incoming viral particles in cells where cholesterol homeostasis is impacted by PF-429242 or mevastatin.

As part of Specific Aim 1 we proposed utilizing modified haploid cells (HAP1) that constitutively express the LDL receptor (LDLR) so as to identify additional entry factors, unrelated to cholesterol metabolism, for ANDV (Major Task 3). Toward this end a viral vector expressing LDLR was constructed (Figure 4) and used to produce HAP1 cells constitutively expressing LDLR. The

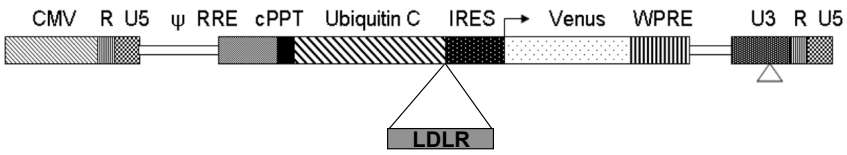


Figure 4. Vector for production of HAP1 cells constitutively expressing LDLR. *The LDLR cDNA was inserted into the FCIV (FM5) lentiviral vector (generous gift of Dr. Jeffrey Milbrandt lab, Washington University). This vector uses the ubiquitin promoter to express the gene of interest and also expresses the Venus fluorescent protein via an internal ribosome entry site. The sequence and orientation of the insert was verified by complete sequencing. Lentivirus (FCIV-LDLR) was produced by transient co-transfection of 293T cells with psPAX2 and pCMV-VSV-G then used to transduce HAP1 cells.*

haploid, Venus+ cells were isolated by FACS sorting. We had previously confirmed by flow cytometry of cell surface staining with an LDLR antibody that as expected LDLR is co-expressed in Venus+ cells (data not shown). We used the smaller size of haploid cells by side scatter as a gate along with Venus expression for sorting. The selected cells were expanded and characterized by flow cytometry as shown in Figure 5. As can be seen the majority of the cells express Venus (Figure 5A) and upon display a Hoechst staining profile consistent with 59% 1n (haploid) and 40% 2n (diploid or S phase haploid) cells. Notably there is no 4n peak as would be expected for dividing diploid cells. These haploid, LDLR+ cells were then used to construct a LentiRET mutagenized library as described in the original proposal. Unfortunately a cryogenic storage system failure resulted in loss of this library and the HAP1-LDLR parent cells delaying the screening aspect of aim 1. Since that incident last spring we have performed the steps outlined above and re-created the mutagenized library (which is now backed up in several Liquid N2 tanks) and are ready to screen.

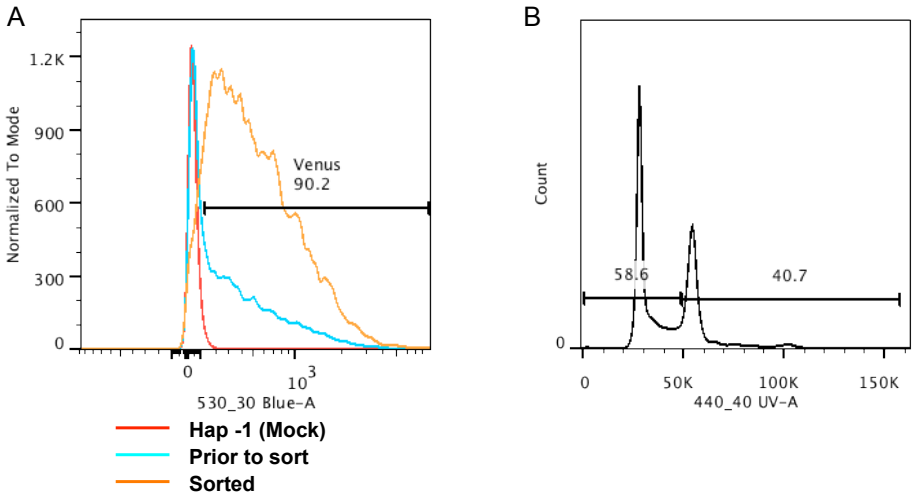


Figure 5. Selection of LDLR expressing haploid cells. *HAP1 cells transduced with FCIV-LDLR were expanded and then 75 million cells were sorted for Venus expression and small size by side scatter. After expansion, the cells were characterized by flow cytometry for A) Venus expression and B) DNA content upon Hoechst staining.*

Specific Aim 2 of this proposal is to utilize haploid cell mutagenesis and screening (4,5) to identify and characterize host factors involved in Old World Hantavirus entry (Major Tasks 4 & 5).

The strategy for this is outlined in Figure 6A. The experimental methods employed for this analysis are essentially the same as those used previously by our lab to identify host factors needed for Andes viral infection (1). A mutagenized HAP1 library containing 75 $X10^6$ independent mutants was screened for survivors of infection by a VSV recombinant virus carrying the PUUV glycoproteins (rVSV-PUUV; MT4 subtask 2). Ten days after infection DNA was prepared from the small number of surviving cells and ligation mediated PCR was performed to selectively amplify the end of the integrated Lenti-RET mutagen and the host flanking DNA. This sequence data was used to map integration sites of the lentivirus mutagen (MT4 subtask 3). Integration sites for the PUUV-selected population were mapped to the human genome and compared to >500,000 integration sites mapped in the starting unselected HAP1 library to identify those mutagenized genes enriched in the selected cells (MT4 subtask 4). Figure 6B shows the results of this analysis with each circle representing a gene identified in the selected cell population. The size of the circle indicates the number of independent integrations (mutations) observed in the selected cells. The orange line denotes p-values less than 0.01. Indicated are four genes (SCAP, MBTPS1, MBPTS2, and SREBF2) of the SRC that were found to be highly enriched in PUUV resistant cells. We have employed genetic and pharmacologic methods to verify the importance of these factors for PUUV infection. We confirmed the requirement for these genes in PUUV infection utilizing genetic knockout cell lines, siRNA and pharacologic inhibitors of this pathway (data not show) thus completing most of the studies described in Major Tasks 4 and 5. A manuscript describing this work is in progress.

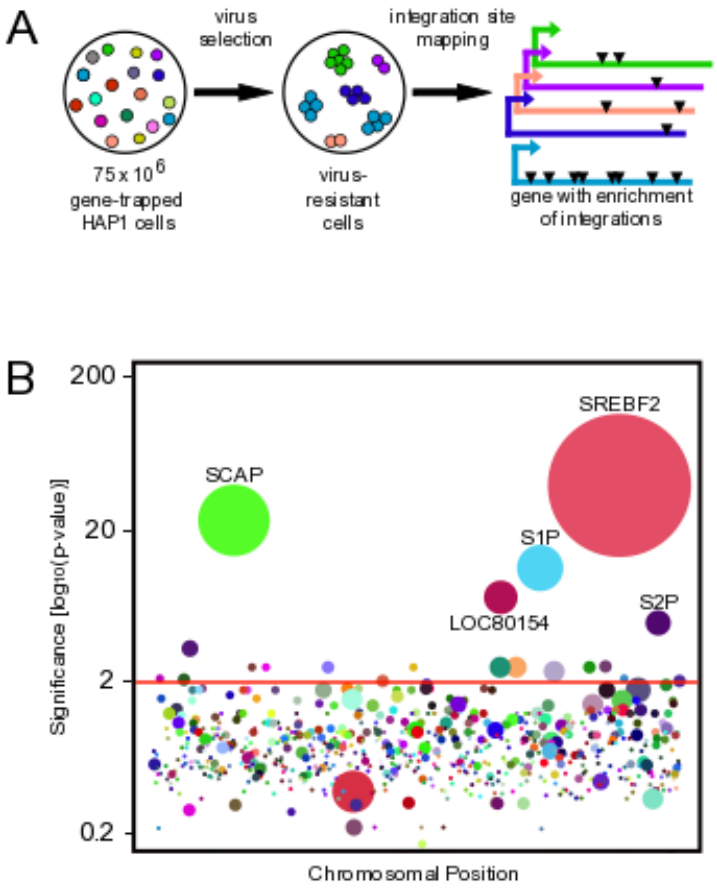


Figure 6. Haploid cell screen for cellular factors required for rVSV-PUUV infection. *(A) A diagram of the HAP1 forward genetic screening process. HAP1 cells were insertionally mutated with LentiRET then selected by infection with recombinant rVSV-PUUV. Following expansion, DNA was extracted and ligation mediated PCR was performed followed by Illumina sequencing. Mapping of the insertion sites of selected cells and unselected cells and comparison of these sites in each sample allowed the identification of genes enriched in integrations in the selected sample relative to the unselected. (B) A dot plot showing the approximate chromosomal position and statistical significance of the genes identified in the HAP1 rVSV-PUUV screen. The gene names of the five most significant hits are noted. The size of the dot indicates the number of unique integrations mapped to that gene, while the orange horizontal line marks a p value of 0.01. P values have been corrected for false discovery rate.*

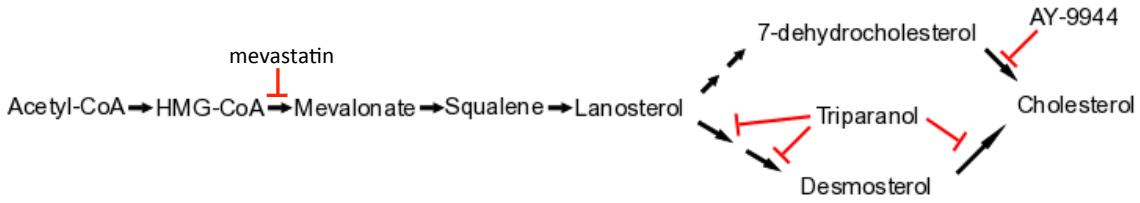


Figure 7. An abridged scheme of the sterol biosynthetic pathway. *The enzymatic steps targeted by the drugs utilized in this study are noted.*

In addition to analysis of the SRC inhibitors on PUUV infection we delved deeper into the cholesterol synthesis pathway to determine if there is a direct requirement for cholesterol or another sterol or by-product. To address this question two inhibitors of steps late in the cholesterol synthesis pathway (see Figure 7, Triparanol & AY-9944) were examined for their effect upon VSV-PUUV infection. As is seen in Figure 8 perturbation of late steps in cholesterol synthesis dramatically reduce PUUV infection. Additionally, entry of the New World hantavirus Andes is also significantly impaired by these compounds. These studies speak to the mechanism of inhibition experiments that are components of Major Tasks 2 and 4.

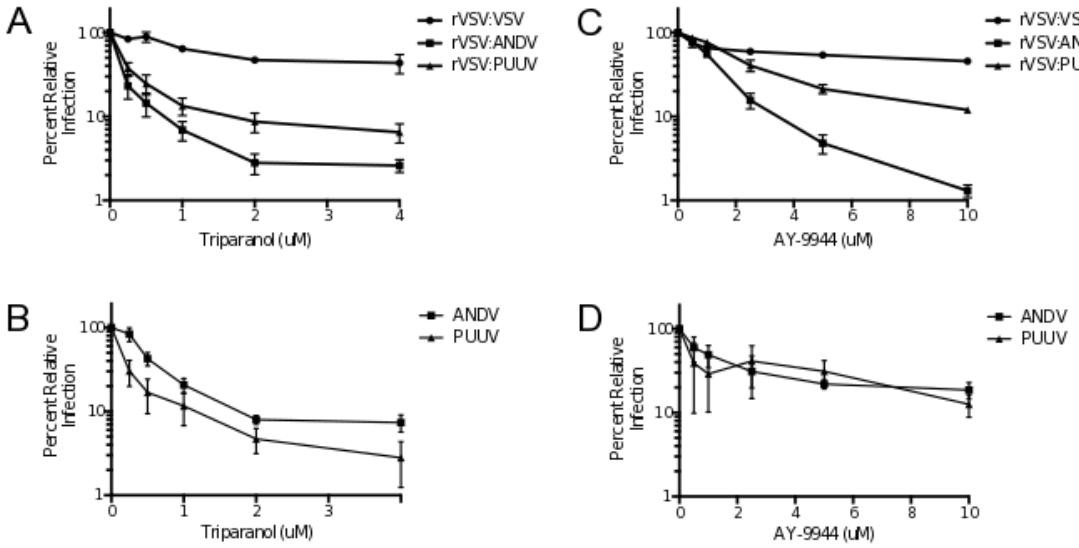


Figure 8. Disruption of late events in the cholesterol biosynthetic pathway inhibits hantavirus entry. *(A) and (B) Inhibition of reactions downstream of lanosterol decreases PUUV entry. A549 cells were cultured in delipidated media with vehicle or increasing concentrations of triparanol, an inhibitor of various reactions in the desmosterol-containing branch of the cholesterol biosynthesis pathway. Cells were subsequently infected with (A) recombinant VSV expressing PUUV, ANDV, or VSV glycoproteins, or (B) wild type PUUV or ANDV. Infection was quantified by flow cytometry and normalized to vehicle controls. N=6 Bars=SEM. (C) and (D) Inhibiting the conversion of 7-dehydrocholesterol to cholesterol decreases PUUV entry. A549 cells were pretreated with vehicle or increasing concentrations of AY-9944, which inhibits the production of cholesterol from 7-dehydrocholesterol. Cells were then infected with (C) recombinant VSV expressing PUUV, ANDV, or VSV glycoproteins, or (D) WT PUUV or ANDV. Infection was quantified by flow cytometry and normalized to vehicle controls. N=6 Bars=SEM.*

The goal of specific aim 3 is to identify and characterize factors needed for a second New World Hantavirus (Sin Nombre; SNV) so as to compare to the requirements we have described for Andes virus. All of the work for this aim is scheduled for year 2 & 3 (Major Tasks 7-9).

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- What opportunities for training and professional development has the project provided?
- "Nothing to Report."
- How were the results disseminated to communities of interest?
- Results of the initial experiments analyzing Puumala virus were presented by Ben Dyer in July at the 2015 American Society for Virology Meeting in Ontario, Canada
- What do you plan to do during the next reporting period to accomplish the goals?
- In the short term we are working to re-create the HAP1 lenti and HAP1/LDLR Lenti libraries to allow screening for additional host factors (Major Tasks 3 and 7). This year we expect to use the HAP1 cells expressing LDLR to allow us to delve deeper into host requirements. Also, we will begin the microscopy experiments to determine how these inhibitors of entry work (Major Tasks 2 & 6). Finally, we are currently drafting the manuscript describing the Puumala virus screen and

results ands expect to submit this for publication before the end of 2015.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

- What was the impact on the development of the principal discipline(s) of the project?
- These are the first forward genetic experiments designed to elucidate cellular factors needed for hantaviral entry into host cells. Thus the knowledge gained here will not only inform the basic biology of hantaviral replication but should also describe potential inhibitors of hantaviral infection. Additionally, we have developed replication-competent recombinant viruses that carry the hantaviral glycoproteins but are safer and can therefore be handled in lower biosafety levels. Although we developed them to facilitate genetic screening, these viruses should be of utility to other groups developing hantaviral infection inhibitors and vaccines.
- What was the impact on other disciplines?
- "Nothing to Report."
- What was the impact on technology transfer?
- "Nothing to Report."
- What was the impact on society beyond science and technology?
- "Nothing to Report."

5. CHANGES/PROBLEMS: As described in the report, a cryogenic storage system failure has modestly delayed our progress. We have already begun re-generating the lost libraries and expect to "catch-up" this year.

- Changes in approach and reasons for change
- A very minor change is that we are utilizing a new technology (APEX) that allows us to more effectively perform the studies on hantaviral entry and the mechanism of various inhibitors. Overall this change is of minimal impact on the design of the proposed experiments.
- Actual or anticipated problems or delays and actions or plans to resolve them
- In June 2015 a cryogenic storage unit failed resulting in the loss of a large HAP1 lentiviral mutagenized library and also a new LDLR expressing HAP1 library. This set back experiments described in Major Task 3 and will impact Major Task 7. Using backup HAP1 cells from a separate cryogenic storage facility we will re-construct these reagents this year.
- Changes that had a significant impact on expenditures
- Nothing to Report
- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
- Nothing to Report
- Significant changes in use or care of human subjects
- Nothing to Report

- Significant changes in use or care of vertebrate animals.
- Nothing to Report
- Significant changes in use of biohazards and/or select agents
- Nothing to Report

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- Publications, conference papers, and presentations
- Journal publications. NA
- Books or other non-periodical, one-time publications. NA
- Other publications, conference papers, and presentations.
- Presentation entitled Discovery of "Host Factors Required for Hantaviral Infection" by Ben Dyer (student) at the 34th Annual Meeting of the American Society for Virology. July 2015.
- Website(s) or other Internet site(s) NA
- Technologies or techniques We have generated the technology to produce lentiviral mutagenized HAP1 libraries that can be used to identify important genes for numerous cellular processes. This technology will be shared freely with the scientific community.
- Inventions, patent applications, and/or licenses NA
- Other Products We have generated recombinant VSV viruses that carry various hantaviral glycoproteins. These viruses will be of general utility to groups analyzing hantaviral entry and/or inhibitors of hantaviral infection including antibodies and vaccines. Additionally, we have created cell lines in which genes encoding SRC factors have been inactivated by CRISPR technology which will be of utility to others who work in this area. These reagents will be shared freely with the scientific community
- PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
- What individuals have worked on the project?

▪ Name:	Paul Bates
▪ Project Role:	PI
▪ Researcher Identifier (e.g. ORCID ID):	PFBATES (eRA Commons ID)
▪ Nearest person month worked:	12
▪ Contribution to Project:	Directed Project, coordinated team
Funding Support:	NIH R01AI081913, P51OD011104-53S1, this award
▪ Name:	MJ Drake
▪ Project Role:	Graduate Student
▪ Researcher Identifier:	MJDRAKE (eRA Commons ID)
▪ Nearest person month	12

worked:	
▪ Contribution to Project:	Performed experiments, analyzed data
Funding Support:	NIH R01AI081913, NIH T32AI007324, this award
Name:	Natalia Shalginskikh
Project Role:	Postdoctoral Fellow
Researcher Identifier:	
Nearest person month worked:	11
Contribution to Project:	Performed experiments, analyzed data
Funding Support:	This award
Name:	Ben Dyer
Project Role:	Graduate Student
Researcher Identifier:	
Nearest person month worked:	12
Contribution to Project:	Performed experiments, analyzed data
Funding Support:	This award, NSF Fellowship
Name:	Steven Bart
Project Role:	Graduate Student
Researcher Identifier:	SMBART (eRA Commons ID)
Nearest person month worked:	3
Contribution to Project:	Performed experiments, analyzed data
Funding Support:	This award, Institutional Funds, NIH T32AI007324

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- Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
- The R01 grant that supported a portion of Dr. Bates effort will terminate this year.
- What other organizations were involved as partners?
- Nothing to Report.

7. SPECIAL REPORTING REQUIREMENTS

- COLLABORATIVE AWARDS:
- Nothing to Report.
- QUAD CHARTS (revised): Attached

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APPENDICES: SOW (revised) - Attached